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STUDIES ON THE LINKS BETWEEN WHITE ADIPOSE TISSUE PHENOTYPE AND THE CIRCULATORY SYSTEM

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Studies on the Links Between White Adipose Tissue Phenotype and the Circulatory System

Thesis for Doctoral Degree (Ph.D.)

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ABSTRACT

Obesity is a strong risk factor for cardiovascular disease. It is defined by an unhealthy excess of white adipose tissue (WAT). Adipose derived weight gains and losses are linked to changes in two other independent cardiovascular risk factors: hypertension, and arterial stiffness. The aim of this thesis was to explore which specific WAT factors associate with hypertension and arterial stiffness.

Subjects undergoing bariatric surgery were utilized as a model system for linking different WAT conditions with vascular outcomes. Phenotyping methods included anthropometric measurements, hyperinsulinemic euglycemic clamp, dual energy Xray absorptiometry assessment of WAT distribution and adipose tissue biopsies for RNA sequencing and *in vitro* assessments of adipocyte function and characteristics.

Study I assessed the cross-sectional relationships between WAT factors in obese individuals (n = 120) and pulse wave velocity (PWV), a measure of arterial stiffness. After adjusting for relevant confounders, only visceral adipocyte volume was significantly (and positively) associated with PWV. **Study II** determined if weight loss resulted in long term reductions in PWV and if any WAT factors could predict improvement. The same individuals, except those on antihypertensive pharmacotherapy, were included (n = 82). Subjects were reassessed two years after bariatric surgery and attained a pronounced weight reduction and a significant reduction in PWV. After adjusting for confounders, subcutaneous adipocyte volume and WAT *COL4A1* gene expression was found to predict improvements in PWV. **Study III** investigated which WAT factors associated with blood pressure improvements after weight loss. Here, only hypertensive subjects who underwent RNA sequencing (both at fasting and in hyperinsulinemic state, after a two-hour hyperinsulinemic euglycemic clamp) were included, ten who improved and eight who did not. WAT insulin sensitivity (defined as WAT insulin induced transcriptomics) was the only WAT factor that differed between the two groups, with both a larger response seen at baseline and a larger increase after weight loss observed in the improved group compared to the non-improved group. Finally, perturbed adipose tissue lipolysis is a hallmark of WAT in obesity, contributing to pathophysiological whole-body effects. However, the effects of obesity on atrial natriuretic peptide (ANP) stimulated lipolysis, have never been studied. In **Study IV** ANP stimulated lipolysis was impaired *in vitro* in obesity (n=87) but normalized after weight loss (n = 52). Furthermore, ANP stimulated lipolysis was attenuated *in situ* in overweight men (n = 9). Protein analysis found obese WAT to have a lower expression of NP receptor A which may explain the blunted response.

In summary, studies in this thesis have contributed to the characterization of specific WAT factors that associate with circulatory system pathology and, for the first time, characterized the ANP stimulated lipolytic response in obesity.

LIST OF SCIENTIFIC PAPERS

I. Arner P, **Bäckdahl J**, Hemmingsson P, Stenvinkel P, Eriksson-Hogling D, Näslund E, Andersson DP, Caidahl K, Rydén M. Regional variations in the relationship between arterial stiffness and adipocyte volume or number in obese subjects. Int J Obes (Lond). 2015 Feb;39(2):222-7.

II. **Bäckdahl J**, Andersson DP, Eriksson-Hogling D, Caidahl K, Thorell A, Mileti E, Daub CO, Arner P, Rydén M. Long-Term Improvement in Aortic Pulse Wave Velocity After Weight Loss Can Be Predicted by White Adipose Tissue Factors. Am J Hypertens. 2018 Mar 10;31(4): 450-457.

III. **Bäckdahl J**, Kwok K, Gao H, Mejhert N, Rydén M Blood Pressure Improvement Following Weight Loss is Predicted by the Transcriptional Response to Insulin in White Adipose Tissue. Manuscript

IV. Rydén M, **Bäckdahl J**, Petrus P, Thorell A, Gao H, Coue M, Langin D, Moro C and Arner P. Impaired atrial natriuretic peptide-mediated lipolysis in obesity. Int J Obes (Lond) 2016 Apr;40(4): 714–720.

CONTENTS

LIST OF ABBREVIATIONS

ANP	Atrial natriuretic peptide
ATGL	Adipose triglyceride lipase
BMI	Body mass index
BP	Blood pressure
CVD	Cardiovascular disease
DXA	Dual energy X-ray absorptiometry
ECM	Extracellular matrix
eNOS	Endothelial nitric oxide synthase
ESAT	Estimated subcutaneous adipose tissue
EVAT	Estimated visceral adipose tissue
FFA	Free fatty acids
HIF-1	Hypoxia inducible-factor 1
HSL	Hormone-sensitive lipase
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
NO	Nitric oxide
RAAS	Renin-angiotensin-aldosterone-system
RYGB	Roux-en-Y gastric bypass
SNS	Sympathetic nervous system
TG	Triglycerides
UCP1	Uncoupling protein 1
PLIN	Perilipin 1
PKA	Protein kinase A
PKG	Protein kinase G
PP	Pulse pressure
PWV	Pulse wave velocity
WAT	White adipose tissue

1 INTRODUCTION

1.1 OVERWEIGHT AND OBESITY

Being overweight or obese is defined by having an excess fat accumulation for a given height. The most common categorization method is by body mass index (BMI), which is calculated by dividing a person's weight in kilograms by their squared height in meters. Values above 25 are termed overweight, above 30 obese, above 35 severe obesity and above 40 morbid obesity. The formula has pitfalls: muscle weighs more than fat, causing e.g., body builders to frequently have an unhealthy BMI which is not due to having excessive fat tissue.

The direct cause of increasing body weight is an imbalance between energy intake and expenditure (Figure 1), which in turn may be due to a wide range of factors. This occurs despite that few desire to be overweight or obese**,** as both most modern-day beauty standards and health views support a lean body type**.**

Our environment has become increasingly obesogenic. Labor used to be manual, forcing us to expend energy to earn a living. Food was generally unprocessed and relatively energy poor. Today, sedentary jobs dominate, while food is available anywhere, and anytime. It is often processed and rich in calories. When served in restaurants portion sizes often widely out scale public health recommendations [1], and it's been shown that humans will consume 500Kcal/day more given an *ad libitum* intake of an ultra-processed diet compared to an unprocessed one [2]. Obesity prevalence has doubled since 1980 [3], in Sweden it is now more common for adults to be overweight and obese compared to lean, [4] and increases in body weight can be seen across the entire population spectrum around the globe.

This obesity epidemic has severe consequences far greater than any perceived cosmetic downsides, with deaths attributed to an excess fat accumulation estimated at 2.8 million per year [3]. Furthermore, it is at least partially responsible for 44% of the diabetes burden, 23% of ischemic heart disease and between 7-41% of different cancer incidences [3]. On average, if you are obese at age 40 your life expectancy will be reduced by roughly four years compared to that of a lean individual [5].

1.1.1 Metabolic syndrome

Excess adipose tissue (especially when located around the midsection) is often associated with dyslipidemia, insulin resistance and hypertension. To prompt awareness and attention, clinicians and researchers coined the term metabolic syndrome; a constellation of risk factors commonly occurring together which greatly increase the risk of type 2 diabetes and cardiovascular disease (CVD). Specific inclusion criteria and cut off values have varied by different defining organizations, but harmonizing attempts have been made and measures now include increased waist circumference (population specific), reduced HDL-C (<1mmol/l), raised triglycerides (TG) (1.7 mmol/l), elevated blood pressure (130 systolic and 85 diastolic mmHg) and elevated fasting glucose (5.6mmol/l) [6].

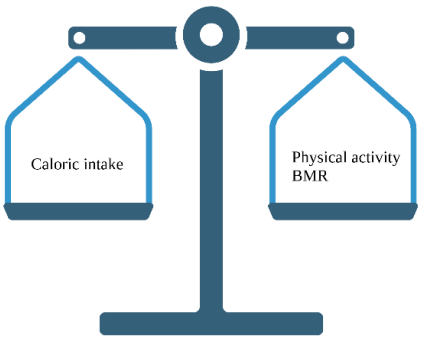


Figure 1. Either a hypercaloric intake or lowering of energy expenditure will lead to increasing body weight. BMR: basal metabolic rate

1.2 OBESITY TREATMENT

Medical treatment of obesity is aimed at reducing and maintaining at least a 5-15% weight reduction, as this has proven to induce significant beneficial effects in obesity-related outcomes [7].

1.2.1.1 Lifestyle treatment

The cornerstone of obesity treatment is lifestyle changes, with reduction in caloric intake and/or an increase in physical exercise. While this strategy results in significant weight loss, the effect is often transient and difficult to maintain. This is demonstrated e.g., by the Look Ahead trial which over a 10-year follow-up showed a 6% body weight reduction in the intervention group vs a 3.5% in the control group. The trial was stopped after 10 years as there was no difference in cardiovascular events between the groups [8]. In line with this, the recent DIRECT trial found only 11% of the study population were able to maintain a 15kg weight loss at 24 months [9]. Despite the somewhat disappointing effects on body weight, it is clear that lifestyle interventions can prevent new onset cases of diabetes [10, 11].

1.2.1.2 Pharmacotherapy treatment

Pharmacological treatment is currently available in three different forms: orlistat a compound inhibiting intestinal lipases, leading to a 30% reduction in fat uptake; bupropion/naltrexone which reduces food craving (it is rarely prescribed as it can increase suicide and mood disorders); and liraglutide, an incretin analogue, which decreases appetite and food intake. Of these, liraglutide has shown the most promise with the highest proportion of users being able to achieve at least a 5% weight loss at 12 months [12]. In the US, the drug combination of phentermine (an appetite reducing drug) and topiramate, an anticonvulsant, is also registered and can achieve a 5% weight loss [12].

1.2.1.3 Bariatric surgery

Bariatric surgery has been proven more effective compared to non-surgical treatment, in reducing body weight, with subjects losing on average an extra 26 kg [13]. It can also achieve diabetes and hypertension remission [13, 14] and lower mortality [15]. There are several different surgical techniques used today (Figure 2), with the most common ones obtaining comparable short- and long-term results [16, 17]. Today (2017), in Sweden, the most common surgical technique is the Roux-en-Y gastric bypass (RYGB) [18], where a small gastric pouch is connected straight to the jejunum, while the rest of the stomach, no longer connected to the esophagus, with the bypassed duodenum is anastomosed to the jejunum. Gastric sleeve is becoming almost as common. Here, a large part of the stomach is stapled off, leaving only about 10-20% of the original area, but there is no anatomical rearrangement. Other techniques include laparoscopic adjustable gastric banding, where a silicone band is placed in the upper part of the stomach and filled with saline, restricting access to the gastric pouch. It is less popular today, as the band can erode, lose effectiveness, and cause complications such as gastro-esophageal reflux disease. Finally there is the biliopancreatic diversion with duodenal switch, a much more challenging and anatomically altering procedure which, while considered the most effective treatment for severe obesity, is hardly used due to long term nutritional deficiencies and short term complications such as surgical leaks or obstruction [19]. In total about 5400 bariatric surgeries were performed in Sweden in 2017 [18]. It's believed that the weight loss effect comes not only from the restrictive effect of surgery, but also due to a functional and structural change of the gastrointestinal tract leading to alterations in many hormones, particularly incretins (e.g., glucagon-like peptide 1, gastric inhibitory polypeptide). These hormonal changes mainly affect the brain and induce satiety [20].

1.3 ADIPOSE TISSUE

Adipose tissue had long been viewed solely as a storage space for excess calories. Research during the last decades have discovered it to be a complex tissue engaged in cross talk with multiple other organ systems and playing a vital role in energy storage and whole-body metabolic homeostasis. Volume-wise it is comprised of 90% adipocytes: the cell type specialized in storing lipids in the shape of a droplet. Numerically adipocytes make up only about between 20-40% of cell content [21]. The remaining cells are fibroblasts, endothelial cells, adipocyte precursors and different types of immune cells such as macrophages, dendritic-, B- and T-cells.

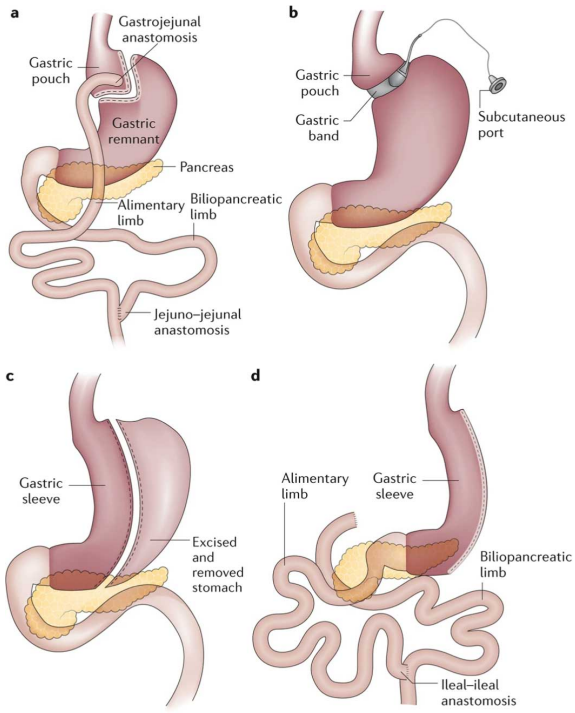


Figure 2. **Bariatric surgery techniques. a** | Roux-en-Y gastric bypass. **b** | Adjustable gastric band. **c** | Sleeve gastrectomy. **d** | Biliopancreatic diversion with duodenal switch. Figure adapted and reprinted with publishers' permission from Nguyen, N.T. and J.E. Varela, *Bariatric surgery for obesity and metabolic disorders: state of the art.* Nat Rev Gastroenterol Hepatol, 2017. **14**(3): p. 160-169.

1.3.1 Brown adipose tissue

Adipocytes can be subdivided based on function into white or brown adipocytes. Brown adipocytes are far outnumbered by white adipocytes. They generate heat through a specific protein, Uncoupling Protein 1 (UCP1), expressed in the mitochondria, white adipocytes lack this protein. Mitochondria store energy as a proton gradient, and as protons flow down their gradient across the mitochondrial membrane they pass through ATP synthase complex, creating ATP. In brown adipocytes there is an alternate route, through an uncoupling protein in the membrane, UCP1, thus the energy dissipates as non-shivering thermogenesis as opposed to creating ATP [22].

Brown adipocytes cluster prominently in specific depots in the neck and scapular areas of young children, while adult humans only have smaller depots throughout the body. Interestingly, cold climate and some pharmacotherapies can induce more brown adipose tissue as well as making white adipose tissue (WAT) "beige" i.e., gaining characteristics of brown adipose tissue [23].

1.3.2 White adipose tissue

White adipose tissue is the main storage site for lipids in the form TGs, which are fatty acid chains attached to a glycerol molecule. Depending on hormonal and neurological stimulation it can take up fatty acids from the bloodstream and increase its lipid storage, a process termed lipogenesis. Upon fasting or during times of starvation, lipids are hydrolyzed through an enzymatic process termed lipolysis, which provides free fatty acids and glycerol as fuel for other tissues.

1.3.2.1 Lipolysis

Lipid droplets are coated with proteins, such as perilipin-1 (PLIN), restricting access from hydrolyzing lipases. Structural changes are required to initiate increased lipolysis in adipocytes. Lipolysis is stimulated by catecholamines and natriuretic peptides and inhibited by insulin. Catecholamines and natriuretic peptides bind cell surface receptors, increasing intracytosolic concentrations of their respective second messengers (cAMP and cGMP) which in turn bind and activate protein kinase A and G (PKA, PKG). These protein kinases phosphorylate hormone-sensitive lipase (HSL), translocating it from the cytosol onto the lipid droplet. Here HSL interacts with PLIN. PLIN is also directly phosphorylated by PKA and PKG, releasing a regulatory component to instead interact and activate another lipase, adipose TG lipase (ATGL). ATGL, HSL and monoglyceride lipase catalyze the hydrolysis of TGs into fatty acids and glycerol which are then either oxidized for energy or released from adipocytes into the bloodstream for use by other organs and tissues. Insulin inhibits lipolysis in part by activating an enzyme, phosphodiesterase 3B, which degrades cAMP.

1.3.2.2 Adipokines

Adipose tissue is a hormonally active tissue. To date several hundred different hormones and cytokines, collectively named adipokines have been discovered. They can be secreted from one or several cell populations within adipose tissue, display a wide variety of effects and affect many different organs and tissues. Notable examples are TNF-α, a pro-inflammatory cytokine attracting leukocytes, inducing insulin resistance and able to stimulate the acute phase reactants in the liver. Adipose tissue TNF-α is believed to act mainly on adipose tissue in a autocrine or paracrine manner, inducing local and systemic insulin resistance in part by increasing basal lipolysis [24]. Adiponectin is exclusively produced in adipose tissue and has many beneficial effects, including increasing skeletal muscle oxidation of fatty acids and decreasing hepatic glucose production [25]. Its expression and serum concentration are inversely correlated with adipose tissue mass. Leptin [26] secretion instead rises with increasing adipose tissue mass and decreases appetite by acting on the hypothalamus.

1.3.3 Adipose tissue depots and distribution

WAT is distributed in different depots in the body. Most of it is located either subcutaneously, under the skin, or viscerally, around the internal organs. There are sexual predispositions as to where excess WAT is stored, with men on average having more visceral

and women more subcutaneous adipose tissue. Hormones likely play a part, as women after menopause become more male-like in their adipose tissue distribution [27]. Glucocorticoids also promote visceral fat accumulation [27].

Excess visceral WAT seems to be more pernicious than subcutaneous WAT. Epidemiological data shows that a larger visceral adipose depot is associated with dyslipidemia and cardiovascular complications [28]. Two main explanations for this have been brought forward: visceral adipose tissue is found near and around the internal organs, and blood flow from the depot drains into the portal system directly onto the liver, and/or there are cell autonomous differences within the different depots [23]. Interestingly, differential gene signatures can be identified between visceral and subcutaneous adipocytes and differences are retained even after several passages in vitro [29]. Lastly, in mice transplanting subcutaneous adipose tissue into a visceral depot improves glucose homeostasis while the reverse has no effect [30].

1.4 INSULIN

Insulin is an anabolic hormone released from specialized cells, beta-cells, in the pancreas in response to increasing blood glucose levels. It has a variety of different effects in various target organs. Thus, in adipose tissue and skeletal muscle, insulin promotes glucose uptake and in skeletal muscle also glycogen synthesis. Furthermore, in adipose tissue, insulin promotes lipogenesis and inhibits lipolysis. In liver insulin also promotes glycogen synthesis and increases de novo lipogenesis while decreasing gluconeogenesis and glucose output.

1.4.1 Insulin signaling

Insulin is a peptide hormone (it cannot pass through cell membranes) that binds a specific cell surface insulin receptor. The insulin receptor is a transmembrane homodimer composed of alpha and beta subunits. Alpha subunits are extracellular and bind insulin. Upon binding, the receptor undergoes a structural change and the intracellular beta subunit, which has tyrosine kinase enzymatic activity, is autophosophorylated and subsequently phosphorylates a target protein known as insulin receptor substrate. This starts a signaling cascade targeting several proteins eventually leading to phosphorylation and activation of AKT, a serine/threonine kinase. AKT has over 100 known substrate targets and mediates most of the physiological effects of insulin, such as glucose uptake (translocation of GLUT 4 to cell membrane), protein synthesis (through mTORC1), de novo lipogenesis (increase in ACLY activity) and decrease in lipolysis (through degradation of cAMP).

After insulin binds its receptor it can either be released back into the circulation and cleared by either the liver or kidney or degraded after endocytosis of the insulin-receptor complex.

1.4.2 Insulin resistance

Insulin resistance can be defined as a condition where cells fail to respond normally to the hormone insulin. Despite decades of research into insulin signaling it is still unclear what perturbations are causative of insulin resistance. Multiple alterations have been described:

decreasing insulin cell membrane receptors, alterations in intracellular signaling cascades, as well as post-translational modifications such as reduced phosphorylation of key insulin modulating effector enzymes. Likely, insulin resistance is a combination of a multitude of these changes.

Insulin resistance often coexists with hyperinsulinemia, as the pancreas adjusts to higher blood glucose levels by releasing more insulin. It is important to note that insulin resistance affects cells and tissues heterogeneously, as an example liver insulin resistance will lead to an inability of insulin to suppress gluconeogenesis but will not affect de novo lipogenesis [31]. Likewise different tissues may be differently affected in selected individuals and there is even evidence that developing insulin resistance in one tissue may have knock-on effects of inducing insulin resistance in other tissues. Examples include high fat diet fed mice where insulin resistance in liver and adipose tissue was found to precede that of muscle [32], and the evidence that lipotoxicity plays a key role in development of both muscle and liver insulin resistance [33].

1.4.3 In vivo insulin resistance measurements

Most commonly, especially in clinical practice, insulin resistance is measured as the inability to adequately decrease blood glucose levels at a given insulin concentration and thus primarily reflects the inability of insulin to suppress gluconeogenesis from the liver as well as GLUT 4 translocation and glucose uptake in adipose tissue and skeletal muscle. Some measurement techniques are discussed below.

1.4.3.1 Hyperinsulinemic euglycemic clamp

The hyperinsulinemic euglycemic clamp is often referred to as the gold standard technique for determining insulin resistance in vivo [34]. It utilizes a continuous high infusion set rate of insulin and a concomitant variable glucose infusion to maintain euglycemia. The amount of glucose infused is termed the metabolized glucose and is expressed per mg/kg/min (M-value). Although not a dichotomous measurement, values around 4.7-4.9 have been proposed as a cut off for labeling an individual insulin resistant [35, 36]. As the high insulin infusion suppresses glucose output from the liver (in order to quantify as precisely as possible how much of the glucose taken up by peripheral tissues is due to the glucose infusion), the hyperinsulinemic-euglycemic clamp primarily reflects muscle and to some extent adipose tissue insulin sensitivity.

1.4.3.2 Homeostasis Model Assessment of Insulin Resistance

The Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) is a mathematical formula used to estimate insulin sensitivity. It is calculated by multiplying fasting plasma glucose (mmol/l) with fasting plasma insulin (mU/l) and dividing the result by a factor of 22.5. Normal HOMA-IR values are around 1, with values over 2.5 deemed insulin resistant [37]. As values for insulin and glucose are measured in the fasting state, HOMA-IR primarily reflects hepatic insulin resistance.

1.4.3.3 Oral Glucose Tolerance Test

The oral glucose tolerance test is a relatively simple method used clinically to diagnose type 2 diabetes or gestational diabetes. The test is performed by having a fasting subject consume 75 grams of liquidized sugar and after two hours have a blood glucose level measured. Values between 7.8-11.1 mmol/l correspond to impaired glucose tolerance and values above 11.1 classify the subject as being diabetic.

1.5 PATHOPHYSIOLOGY OF PERNICIOUS ADIPOSE TISSUE IN OBESITY

Adipose tissue dysfunction impacts whole-body metabolism. This is exemplified by lipodystrophies, a heterogeneous group of disorders characterized by a varying degree of adipose tissue loss despite normal caloric intake. Individuals are prone to both severe insulin resistance as well as hypertension [38]. Similar consequences occur if adipose tissue is subjected to extended periods of hyper-caloric intake. Subcutaneous adipose tissue depots will eventually fail to achieve adequate expansion, and instead fat will be stored ectopically [39] in visceral adipose tissue, liver and skeletal muscle, resulting in metabolic disturbances [40, 41]. Dysfunctional adipose tissue is characterized by several phenotypic changes such as increasing adipocyte size, hypoxia, fibrosis, inflammation and altered lipolysis.

1.5.1 Adipose tissue expansion

There seems to exist a numerical set point of adipocytes in humans which is reached in young adulthood and retained throughout life, despite a yearly turnover of adipocytes [42]. However, when exposed to persistent overfeeding adipocytes will expand both in size and number. In general adipocytes increase first in size and then by number, but this varies between individuals, and some will have proportionally more cells per amount of adipose tissue, hyperplastic adipose tissue, compared to fewer but larger cells, hypertrophic adipose tissue. Larger adipocytes have been linked to a wide variety of pernicious metabolic consequences [43].

1.5.2 Hypoxia and fibrosis

As adipose tissue increases in size and number, depots risk outgrowing their blood supply. Hypoxia inducible-factor 1 (HIF-1), an oxygen sensitive transcription factor, activated by hypoxia, is upregulated in adipocytes in obesity [44], and is linked to metabolic dysfunction [45]. It should be noted that in vivo measurements of obese fat pads show conflicting results as whether there is hypoxia in adipose tissue in human tissue [46], while the correlation is clear in many animal models [47]. Nevertheless, it seems that oxygen tension levels in humans are probably too low for the optimal homeostasis of the adipose tissue depot.

Hypoxia is furthermore believed to lead to fibrosis, which is distinguished by an increase in extracellular matrix (ECM). HIF-1 is deemed to be one culprit, inducing an increased production of ECM [45]. The increase in ECM reduces tissue plasticity, further worsening adipose tissue function. Fibrosis has been closely linked to an increase in immune cell infiltration, additionally promoting inflammation within adipose tissue [45].

1.5.3 Inflammation

Inflammation has long been known to have a role in dysfunctional adipose tissue [48]. Immune cells, especially macrophages, growingly populate obese adipose tissue and give rise to a chronic low-grade inflammation which has been linked to metabolic dysregulation [49-51]. Macrophages have classically been considered to exist in either a more pro-inflammatory state or anti-inflammatory state, but there is also evidence of a macrophage subtype in adipose tissue with a distinct gene expression profile [52]. As adipose tissue mass expands, more pro-inflammatory macrophages populate the fat pad, secreting pro-inflammatory cytokines like TNF- α, IL-6 and IL-1β [53]. While inflammation has been classically linked to insulin resistance and a pernicious WAT phenotype, it is important to note that it also constitutes an important mechanism for adipose tissue remodelling. This has been shown by knocking down different inflammatory pathways in mice, which lead to an inability of visceral adipose tissue to expand during high fat feeding [54], resulting in ectopic lipid accumulation, systemic inflammation, and insulin resistance. Inflammation's key role in adipose tissue remodelling was also shown in humans by blocking il-6 during exercise regimes, which led to the intervention group not losing visceral adipose tissue mass [55].

1.5.4 Lipolysis in obesity

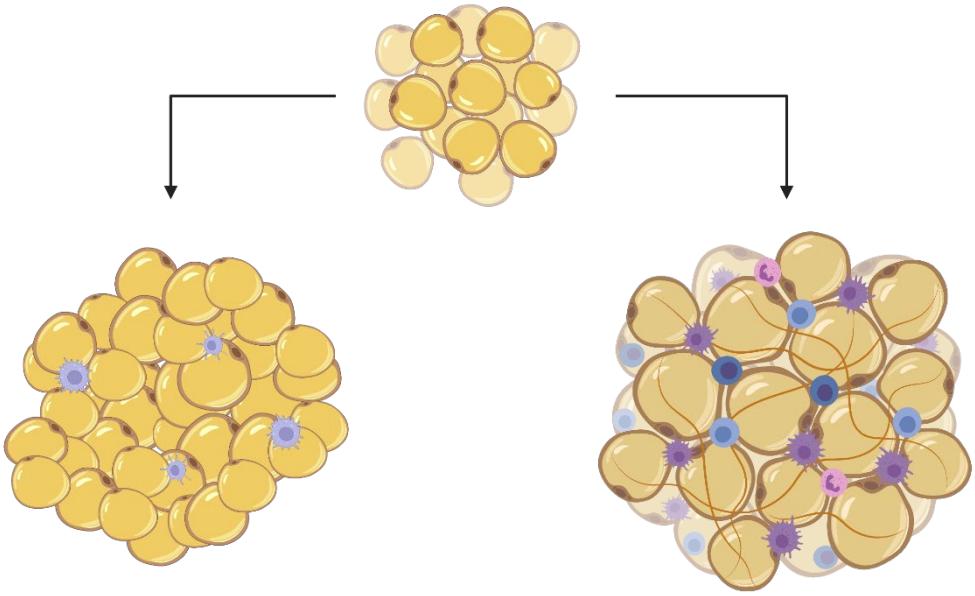
Lipolysis is altered in obese adipose tissue. It is characterized by an increase in basal lipolysis, in part due to increased inflammation which has been shown to act on multiple intracellular pathways decreasing the effects of insulin signaling, downregulating lipid coating protein perilipins (increasing access for TG hydrolyzing enzymes) and stimulating HSL [56]. On the contrary, stimulated lipolysis is blunted [57]. Overall, the net effect in obese adipose tissue is an increase of free fatty acids released into the bloodstream, which gives rise to insulin resistance as they have lipotoxic effects on both liver and skeletal muscle [57, 58].

1.5.5 Impact on whole-body metabolism

While WAT only totals about 5% of postprandial glucose uptake, it has a much larger role in regulating whole-body glucose metabolism through its effect on liver and skeletal muscle. With excess WAT accumulation, and failure to further expand, adipose tissue can no longer properly store TG, leading to ectopic TG deposition in both liver and skeletal muscle, hallmarks of insulin resistance [59]. The increased basal lipolysis (and insulins inability during conditions of insulin resistance to suppress stimulated lipolysis) also gives rise to an excess amount of FFA and glycerol which makes its way to the liver promoting gluconeogenesis [58], non-alcoholic fatty liver disease and lipotoxic metabolites, further exacerbating hepatic insulin resistance [60]. FFA also inhibits insulin-stimulated glucose uptake and glycogen production in muscle and instead promotes lipid synthesis [60].

1.5.6 Pernicious adipose tissue summary

The pernicious enlarged adipocyte, hypoxic, fibrotic, and immune cell rich adipose tissue with chronic low-grade inflammation signals the end of a healthy adipose tissue depot. It is no longer able to keep up with the constant stress of hyper-caloric intake, and this impacts negatively on whole-body metabolism. Healthy and unhealthy adipose tissue expansion is summarized in Figure 3.



Hyperplastic expansion	**Hypertrophic expansion**
↓ Cell number	**↑ Cell number and size**
↓ Immune cells	**↑ Immune cells**
↓ FFA release	**↑ FFA release**
↓ Hypoxia and fibrosis	**↑ Hypoxia and fibrosis**
↓ Inflammation	**↑ Inflammation**
↑ Insulin sensitivity	**↓ Insulin sensitivity**

Figure 3. Healthy (left) and unhealthy (right) adipose tissue expansion. Figure modified and reprinted with publishers' permission from Choe SS et al. *Adipose Tissue Remodeling: Its Role in Energy Metabolism and Metabolic Disorders* Front Endocrinol (Lausanne). 2016 Apr 13;7:30

1.6 HYPERTENSION

1.6.1 Blood pressure

Blood pressure (BP) is the force applied by the pulse wave on the arterial wall and is expressed as a ratio of systole (contracting heart) and diastole (relaxing heart). BP is determined by the cardiac output, total vascular peripheral resistance and circulating blood volume. This, in turn, is affected by several regulatory systems including sympathetic nervous system (SNS) activation, various hormonal systems such as renin-angiotensin-aldosterone system (RAAS) and natriuretic peptides, sodium intake and the endothelium itself [61]. Perturbations in any or a combination of these factors can result in systematically elevated blood pressure levels. These factors are discussed below. Blood pressure also tends to increase with age, in part due to increasing arterial stiffness and a cumulative exposure to pro-hypertensive factors.

Finally, blood pressure levels measured in isolated human populations remain at 115/75 mmHg during the entire lifespan but increase if individuals migrate [62, 63]. It is likely that blood pressure levels seen in these isolated societies represent what should be considered normal when avoiding exposure to modern day living standards, which includes weight gain, an unhealthy diet, and a sedentary lifestyle.

1.6.1.1 Sodium intake

High serum sodium concentration promotes fluid retention which increases intravascular blood volume and thereby BP. Normally, compensatory mechanisms occur to maintain a normal blood pressure, such as an increase in pressure natriuresis (enhanced sodium excretion by kidneys in response to an increase in perfusion pressure by the increased blood volume) and a decrease in vascular tone (due to an increase in the ratio of endothelium vasodilators to vasoconstrictive factors). Individuals with e.g., endothelial dysfunction (impaired vasodilation in response to stimuli) and a dysfunctional RAAS can become salt sensitive resulting in an increase of systolic BP with high salt intake [64].

1.6.1.2 Natriuretic Peptides

Atrial natriuretic peptides (ANP) are stored and released from the cardiac atria in response to increasing blood volume, which is sensed by stretch receptors in the atria. ANP lowers BP by three mechanisms: vasodilation, increased vascular permeability (shifting fluid to the interstitial compartment away from the intravascular space) and increased renal excretion of sodium and water [65].

1.6.1.3 Endothelium

The endothelium is essential in controlling total peripheral resistance by controlling the contractile state of the arterial tree. There is a continuous secretion of both vasodilators (e.g., nitric oxide (NO) and prostacyclin) and vasoconstrictors (e.g., endothelin 1) in response to blood flow and induced shear stress. Most cardiovascular risk factors can transform the

normal endothelium function to become dysfunctional by induction of inflammation and reactive oxygen species [66] and shifting the balance of vasodilators to vasoconstrictors in favor of the latter in hypertension [67].

1.6.1.4 Renin-angiotensin-aldosterone system

The kidneys play a central role in BP regulation. When blood flow is reduced to the afferent arterioles to the kidneys, specialized cells (juxtaglomerular cells), release and cleave a hormone, prorenin to the active renin. Renin cleaves angiotensinogen, released from the liver, in the bloodstream, into angiotensin I. Angiotensin I is then activated by angiotensin converting enzyme, primarily located in the vascular endothelial cells of the lungs, into angiotensin II. The net effect of angiotensin II is an increase in systemic blood pressure through a variety of effects. There is an increase in total body sodium (by an increase in reabsorption in the proximal convoluted tubules in the kidney through an increase in activity of a sodium-hydrogen exchanger) and from an increase release of aldosterone from the adrenal glands (which inserts luminal NA-channels leading to more sodium absorption). It also induces an increase in peripheral vascular resistance by binding angiotensin type I receptors inducing smooth muscle cell contraction and vasoconstriction.

1.6.1.5 Sympathetic nervous system

Arterial mechanoreceptors, baroreceptors, are placed along the arterial tree (e.g., the carotid sinus), and can sense changes in blood flow. When stretched they signal the brain and inhibit SNS activation through changes in neurotransmitters epinephrine (from the adrenal medulla) and norepinephrine (from nerve terminals). They target adrenergic receptors in arteries (α1 vasoconstrictor and α2 vasodilatory) and the heart (β1 and β2, primarily affecting heart rate) [68]. Moreover, SNS activation of kidneys contributes to sodium reabsorption [69].

1.6.2 Classifying hypertension

Systemic arterial hypertension (hereafter referred to as hypertension) is defined by systematically elevated blood pressures over a defined cut off value. Actual values depend on measurement method and guideline. The most recent European guidelines (published by European Society of Cardiology and European Society of Hypertension) for classifying blood pressure and hypertension are summarized in Table 1. In general, a diagnosis of hypertension should be made using two or more measurements at two or more different time points. Strict cut-offs are somewhat arbitrary, as values above even 115/75 confer an increased CVD risk [61], but they help inform decision making in clinical practice. Guidelines also emphasize the possibility of using ambulatory blood pressure measurements or home blood pressure measurements as they may reveal individuals with white coat hypertension (isolated office elevated measurements) or masked hypertension (normal office blood pressure readings but high out of office measurements).

	Systolic	Diastolic
Optimal	<120	<80
Normal	120-129	80-84
High normal	130-139	85-89
Grade 1 hypertension	140-159	90-99
Grade 2 hypertension	160-179	100-109
Grade 3 hypertension	≥ 180	≥ 110
Isolated systolic hypertension	≥ 140	<90

Table 1. European Society of Cardiology and European Society of Hypertensions guidelines for classifying hypertension. All systolic and diastolic values are measured in mmHg.

1.6.3 Epidemiology

Hypertension is common, with an estimated adult worldwide prevalence of over 30% [70]. It is the single most important risk factor for cardiovascular disease (CVD) and all-cause mortality [71].

Hypertension can be classified as either primary or secondary, depending on the cause. Primary hypertension is a result of complex interactions between genes and environment. Over 120 different loci have been associated with hypertension [72], but explain very little of the variance. Secondary hypertension can be attributed to a specific identifiable cause. They include rare monogenic diseases, hormonal emitting tumors or adrenal diseases, renal artery stenosis, thyroid disorders, obstructive sleep apnea or drug side effects. Primary hypertension constitutes around 90% of cases.

1.6.4 Hypertension treatment

Hypertension treatment is based on removing or modifying lifestyle factors, and pharmacotherapy. The decision to initiate treatment depends on blood pressure levels, age, and future cardiovascular risk. Latest joint guidelines from European Society of Cardiology and European Society of Hypertension recommend initiating both lifestyle changes and drug treatment in subjects with grade 1 hypertension and above. They also advocate for subjects with high normal blood pressure levels who are close to 140/90 mmHg to initiate pharmacotherapy if lifestyle changes fail to lower blood pressure [73]. For younger individuals with grade 1 hypertension and low cardiovascular risk, there is lacking randomized control trial evidence regarding the effectiveness in reducing mortality and morbidity with pharmacotherapy. However, many clinicians find it reasonable to initiate medical therapy, especially if lifestyle changes fail to lower blood pressure.

Proven effective lifestyle changes are weight loss, reduced sodium intake, moderating alcohol intake, smoking cessation, regular physical exercise and keeping a healthy diet [74-76].

Meta-analysis of placebo-controlled trials have established reductions in blood pressure levels and in cardiovascular mortality and morbidity for five drug classes (beta blockers, calcium channel blockers, angiotensin converting enzyme inhibitors, angiotensin II receptor

blockers and diuretics) as equally effective, and should form the basis of hypertension pharmacotherapy treatment [76-79]. While minor differences do exist regarding disease specific improvements and/or risk reductions [76], the overall effect can be attributed to blood pressure levels reduction. However, careful consideration should still be given regarding individual co-morbidities and side effects which can influence drug of choice. Other drug classes have less randomized clinical trial evidence, or more adverse effects and should generally be considered as add on therapies in individuals failing to meet blood pressure goals.

1.6.5 Obesity and hypertension

The link between obesity and hypertension is well established and results from the Framingham heart study suggests that roughly 70% of primary hypertension can in part be attributed to excess weight gain [80]. It is important to note that excess weight gain increases blood pressures across the entire spectrum [81]. Finally, weight loss reduces blood pressure in many subjects [82, 83].

There are several mechanisms by which obesity contributes to hypertension. Firstly, obesity is often accompanied by insulin resistance and hyperinsulinemia, which contributes to hypertension. Possible mechanisms will be discussed below. Secondly, several morbidities and behaviors which can give rise to increased blood pressure and hypertension are commonly seen in obesity. These include sleep apnea, increased dietary salt- and alcohol intake and a reduction in physical exercise.

Lastly, there are obesity- and adipose tissue-specific effects. These include SNS activation which has been reported to be higher in obese subjects [84, 85]. Although obese individuals display an increase in heart rate, this seems more related to a decrease in parasympathetic activity [86, 87]. Obesity is also characterized by an increase in both extracellular volume and blood flow. The blood flow increase is seen in adipose tissue but also other tissues such as skeletal muscle, kidneys and heart [88]. Pressure natriuresis is impaired in obesity due to a higher resorption of sodium and increase in extracellular fluid volume. This, in turn, is likely due to different factors: an increase in SNS specifically in the renal nerves leading to renin secretion and sodium resorption, visceral adipose tissue compressing kidneys and activation of the RAAS [81]. RAAS activation can be due to kidney compression by adipose tissue but there is also RAAS production in the adipose tissue [89]. However, there have been no confirmatory studies to date suggesting it would have causative roll in blood pressure changes. Intake and infusion of fat has been linked to sensitizing peripheral alfa-1 adrenergic stimulation and increased vascular tone [90, 91]. Natriuretic peptides have also been found to be lower with increasing BMI [92]. Lastly, there seems to be an inadequate release of natriuretic peptides in response to volume loading in individuals with metabolic syndrome [93].

1.6.6 Insulin resistance and hypertension

The insulin receptor is expressed in vasculature [94], where it has been shown to stimulate the production of NO (a potent vasodilator), by phosphorylating endothelial nitric oxide synthase (eNOS), increasing its activity [95]. This pathway is likely inhibited in the setting of insulin resistance. It also stimulates the production of endothelium-1, a potent vasoconstrictor and this effect may be even more pronounced in insulin resistance [96]. Insulin has been found to increase vasoconstriction after administration in the setting of severe insulin resistance [97]. Chronic hyperinsulinemia has also been reported to impair the otherwise vasodilatory action of insulin [98] and not improve endothelial function [99].

Obesity is associated with a change in adipokine secretion profile. Many of these could play a role in the development or maintenance of hypertension. For example leptin secretion is augmented in obesity and chronic infusion in rodents was followed by an increase in blood pressure [100]. However, there was no change when administered in humans [101]. Adiponectin secretion diminishes with increasing adiposity and adiponectin has been shown to restore eNOS [102]. Inflammation has been linked to hypertension and a hallmark of obese adipose tissue is immune cell infiltration. These immune cells release many pro-inflammatory cytokines, for example TNF-α, MCP-1 and IL-6, which are believed to play a role in hypertension [103]. In keeping with this, TNF-α antagonist treatment has been shown to hinder the development of hypertension in fructose challenged rodents [104]. Here, the perivascular adipose tissue may be of extra importance due to its anatomical location; it can be postulated that any detrimental changes to this depot in obesity will highly influence vascular health. It has been suggested that this adipose site has a different expression profile compared to other adipose tissue depots [105], and that there is a significant amount of cross-talk between PVAT and blood vessels with reciprocal effects on both tissues [106].

1.7 ARTERIAL STIFFNESS

The reduced arterial capacity to expand and contract with given changes in volume is termed arterial stiffness. Healthy vessels distend when the systolic pulse wave hits the arterial wall and then contract in the diastolic phase. This leads to a decrease in systolic pressure and increase in diastolic pressure. In stiff arteries the capacity for distention and contraction is limited (Figure 4), and instead there is widening of the pulse pressure, the difference between systolic and diastolic pressure. Furthermore, with increasing pulse wave velocity, waves reflected from peripheral circulation (mainly from bifurcations) return while the heart is still in systole, adding pressure to the systolic phase. This results in the left ventricle working harder against a higher pressure, increasing oxygen demand, leading to left ventricle hyperplasia. Stiffening also leads to arterial wall remodeling, causing stenosis and plaque build-up [107]. Arterial stiffness is an independent risk factor for CVD and all-cause mortality [108].

The simplified molecular basis of arterial stiffness is a disturbance in the ratio between the two most important scaffolding proteins in the arterial vessel wall, collagen, and elastin.

Normally the relative contribution of these two proteins is held stable but due to a pro-inflammatory environment there is an abnormal production of the stiffer collagen and a diminishing abundance of elastin, limiting the distensibility of the vessel [109]. There is a thickening of intima-media and infiltration of vascular smooth muscle cells, immune cells, increased matrix metalloproteinases, and cytokines [110, 111]. The stiffening is often patchy and occurs mainly in central arteries [112]. Known risk factors affecting arterial stiffness are age, pulse, blood pressure (note, arterial stiffness is a risk factor for hypertension and arterial stiffness precedes hypertension, but can then be exacerbated by high blood pressure) [113]. Finally, smoking and physical exercise also play a part [114, 115].

1.7.1 Measuring arterial stiffness and vascular health

The easiest and most accessible way to assess arterial stiffness is to measure the pulse pressure, which is the difference between systolic and diastolic blood pressure. Only a normal blood pressure device is necessary. One drawback is that peripheral measured pulse pressures can differ up to 20 mmHg from central pulse pressures [116].

Another non-invasive method is to measure the speed of the pulse wave between two points and divide the result with the travel time. The result is pulse wave velocity (PWV). Arteries that cannot extend during systole will result in an increased speed of the pulse wave travelling down the circulatory system, the stiffer the artery the faster the PWV. Most commonly this is measured either by different pressure sensor devices or doppler probes placed at one or different points in the body, but it is also possible to use oscillometric measures which capture both the systolic and reflected wave to calculate PWV. PWV measures have been used in many studies to predict long term consequences of arterial stiffness, and aortic PWV is an independent predictor of a variety of outcomes in different populations [107]. The PWV between the carotid and femoral artery has been deemed the gold-standard measurement of arterial stiffness [107].

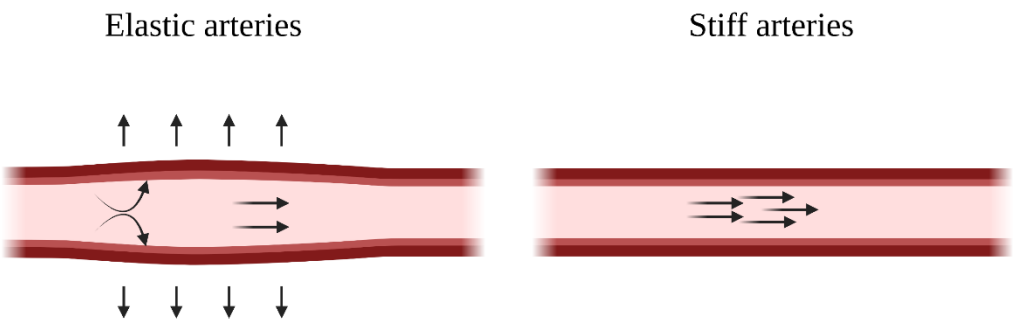


Figure 4. Elastic and stiff arteries. Elastic arteries (left) expand upon force from the systolic pulse wave, lowering systolic blood pressure and reducing the speed of the pulse wave. In stiff arteries (right) the elasticity is reduced resulting in a faster pulse wave. Figure created with biorender.com

1.7.2 Obesity and arterial stiffness

Body weight gain increases arterial stiffness [117]. Short, moderate, and long-term weight loss have all been found to improve arterial stiffness [118-121]. Several studies have explored the underlying causes. Positive associations between arterial stiffness and several adipose and obesity factors have been found: adipokine secretion from WAT [122], insulin resistance [123], WAT distribution [124], inflammation [125], visceral adipocyte volume [126] and circulating levels of free fatty acids [127] have been reported in cross sectional settings. Furthermore, associations between carotid–femoral PWV and Hemoglobin A1c levels, [128] and between aortic PWV and central obesity, plasma adiponectin, and TG levels have been seen in longitudinal observational studies [129]. Taken together, these studies suggest a possible causal relationship between perturbed WAT and arterial stiffness.

Candidate gene- and genome-wide association studies have determined genes whose expression associates with arterial stiffness [130]. In obese WAT one of these genes' expression, *COL4A1*, was associated with improvement in aortic PWV following extensive weight loss [121].

1.8 CONCLUDING REMARKS

Obesity is a large and growing public health concern. It's characterized by an expanding adipose tissue mass which often is dysfunctional and linked to both metabolic and vascular disease. The mechanisms are complex but once understood should hopefully offer further avenues of therapeutic intervention

2 RESEARCH AIMS

The overarching aim of this thesis is to establish which specific WAT factors associate with arterial vascular disease and to investigate the lipolytic effect of ANP in obese WAT.

2.1 SPECIFIC AIMS

2.1.1 Study I

To determine the relationship between arterial stiffness and anthropometric measurements and adipocyte volume and number in obesity.

2.1.2 Study II

To establish if weight loss leads to long term improvement in arterial stiffness, and if any WAT factors can predict changes in PWV after significant weight loss.

2.1.3 Study III

To determine if any WAT factors differ between subjects who improve in BP and those who do not, following significant weight loss.

2.1.4 Study IV

To investigate if the lipolytic response of ANP in WAT is altered in obesity.

3 MATERIALS AND METHODS

3.1 STUDY DESIGN AND SUBJECTS

Participants for all studies were from the NEFA (Non-Esterified Fatty Acid Adipose Factors Behind Insulin Sensitivity clinical trial, NCT01727245) cohort. The NEFA study is an interventional non-randomized clinical trial assessing the effects of weight loss on insulin sensitivity, body composition and vascular function. Participants were obese individuals who voluntarily sought gastric bypass surgery for primarily cosmetic reasons and were recruited from surgical clinics. Also, Study IV included non-obese controls who were recruited from advertisements.

Inclusion criteria was BMI of 30 or above, age between 18 to 60 years, and being scheduled to undergo bariatric surgery by laparoscopic RYGB technique. Exclusion criteria was a diagnosis of diabetes mellitus type 2 with insulin treatment and/or glitazones, oral or parenteral steroid treatment, complicated psychiatric disease, and the use of warfarin.

In Study I we included all obese trial subjects where PWV data was available. In Study II we included all obese individuals where two-year follow-up data was available but excluded individuals on anti-hypertensive medication. In Study III we included all female obese individuals on pharmacotherapy for hypertension as well as female subjects with two-time points measurements over 140 mmHg systolic and/or 90 mmHg diastolic blood pressure at baseline. Finally in Study IV we included all available obese and non-obese women. For this study we also included data from our collaborators in France who recruited a cohort of lean and overweight men.

3.1.1 Bariatric surgery

All obese subjects underwent RYGB surgery at either Ersta or Danderyd Hospital in Stockholm, Sweden.

3.2 SUBJECT PHENOTYPING

All examinations listed below were collected during a single visit (per time point) at our clinical laboratory, which employed the same research nurses and laboratory technicians during the entire trial period. Subjects had been instructed to fast, including caffeine and nicotine, from 22:00 the preceding evening.

3.2.1 Dual energy x-ray absorptiometry

To determine body composition of different adipose tissue depots: android and gynoid regions as well as estimated visceral adipose tissue (EVAT) and estimated subcutaneous adipose tissue (ESAT), subjects underwent a dual energy X-ray absorptiometry (DXA) scan. A GE Lunar iDXA from GE Healthcare, Madison, WI, USA, was used for all examinations. It utilizes a software (enCORE software, version 14.10.022, GE Healthcare, Madison, WI, USA) to determine android and gynoid fat from pre-determined anatomical markers.

Furthermore, we utilized another software (Corescan, GE Medical Systems, Chalfont St. Giles, UK) to determine visceral fat mass in the android region defined by enCORE. ESAT (in the android region) was calculated by subtracting EVAT from total android fat. The use of DXA to determine visceral fat mass has been approved by the US food and drug administration and correlates excellently ($r^2 \geqslant 0.95$) with computer tomography values [131]. Finally, additional advantages are that the method exposes individuals to a very low dose radiation and is easy to use, especially for obese patients.

3.2.2 Hyperinsulinemic euglycemic clamp

As previously mentioned, hyperinsulinemic euglycemic clamp is considered the gold standard *in vivo* measurement of insulin sensitivity, reflecting primarily that of skeletal muscle and to some degree, adipose tissue. We administered a 1,6 units bolus dose of insulin (Actrapid, Novo Nordisk, Copenhagen, Denmark) per square meter of body surface area. This was followed by a continuous two-hour infusion of intravenous insulin at a rate of 0,12 units/m^2 body surface area per minute. Insulin was diluted with 2 ml albumin (200 g/l, Albunorm Octapharma, Stockholm, Sweden), 82 ml sodium chloride (9 mg/ml) and, to avoid hypokalemia, 16 ml potassium chloride (2 mmol/ml). Concomitantly, a variable infusion of glucose (200mg/ml) was administered to keep blood glucose between 4.5-5.5 mmol/L. Blood glucose values were determined from samples drawn from the dorsal side of a hand which kept in a 63 °C heated box. Samples were drawn in pairs, with the average of the two results recorded every fifth minute and analyzed on a Hemocue (Ängelholm, Sweden).

Glucose infusion rates during the second hour of the clamp was used to determine the glucose disposal rates (M-value; mg/kg*min). Since a relatively high insulin dose was administered during the clamp, we assumed that hepatic glucose output was non-existent, and the glucose infusion rate reflected peripheral tissue glucose uptake.

3.2.3 Biochemistry

Fasting blood glucose samples were drawn for routine clinical chemistry measurements. We analyzed: creatinine, glucose, thyroid stimulating hormone, total cholesterol, TGs, high density lipoprotein, apolipoprotein a, apolipoprotein b, aspartataminotransferas, alaninaminotransferas, glutamyltransferas, hemoglobin A1c, calcium, albumin. 25-oh d vitamin, and c-reactive protein. An accredited university hospital laboratory (Karolinska University hospital) performed all the analysis.

3.2.4 Blood pressure measurements

Blood pressure was measured after a 15-minute rest with subjects in the supine position, using an automated device (Omron M10-IT, Omron Health Care, Hoofddorp, the Netherlands). Two measurements were taken, and if they differed significantly, a third measurement was obtained.

3.2.5 Aortic pulse wave velocity

Aortic PWV (hereafter referred to as PWV, as no other PWV was measured in these studies) was recorded with an oscillometric device (Arteriograph, TensioMed, Budapest, Hungary). Cuff size was based on the subject's arm circumference at the time of examination. PWV was calculated by dividing the pulse wave traveled distance (suprasternal notch to the pubic bone), with the transit time (RT/2). Distance was measured in a straight line next to the subject to avoid any influence from the abdominal circumference. Final values were the mean of 3 measurements. Recommended standardized procedures for obtaining pulse wave velocity measurements were followed [132].

The Arteriograph provides other vascular measures that were recorded: augmentation index which is the percentage of central pulse pressure attributed to the reflected wave from the periphery, pulse pressure the difference between systolic and diastolic blood pressure, (PP) and the central systolic blood pressure.

3.2.6 Questionnaire and interview

Subject history with regards to medications, physical exercise and tobacco use was captured by written questionnaire and confirmed by interview. Physical exercise was graded on a four-point incremental scale with 1 = no regular exercise, 2 = light exercise sometimes 3 = regular physical exercise regime several times a week and 4 = near daily physical exercise.

3.2.7 Adipose tissue biopsies

Visceral adipose tissue biopsies were collected during bariatric surgery (from the greater omentum). Subcutaneous adipose tissue biopsies were taken by needle biopsy before (fasting state) and after the two-hour hyperinsulinemic euglycemic clamp (hyperinsulinemic state). Biopsies were taken laterally of the umbilicus after an injection of lidocaine. Using a needle and 10 ml syringe, adipose tissue was extracted by negative pressure. The aspirated fat was rinsed over a plastic filter with sodium chloride. Coagulated blood was manually removed by the use of forceps or plastic spatula. Unlike samples obtained during general surgery, needle aspirations have been shown to not impact on adipose metabolism or lipolysis [133].

3.3 EX VIVO PHENOTYPING

Adipose tissue samples were transported in sodium chloride to the laboratory immediately after biopsies were obtained. At the laboratory samples were washed both in sodium chloride and then with washing buffers (Krebs Ringer Phosphate buffer with 1 % and 0.1% concentration bovine serum albumin). Mature adipocytes were separated from the stromal vascular fraction by incubation in a 37°C water bath with 0,5mg/ml collagenase and 4% bovine serum albumin for 60 minutes. Adipocytes were then washed again (with the previous washing buffer) and subsequently filtered through a nylon filter three times.

3.3.1 Adipocyte measurements

Adipocyte diameter was determined in accordance with previously established methods [134]. A droplet from the adipocyte suspension was placed on a cover slide and examined under direct light microscopy. The diameter of 100 cells were counted with a ruler in the ocular of the microscope. As adipocytes are spheric, the mean adipocyte volume was approximated using a well-established formula [135]. Approximations have been found to correlate well with adipocyte size from sectioned adipose tissue [136].

The average density of TGs (here defined as triolein, a TG formed from glycerol esterified with three oleic acids) is 0.915 g/cm^3 and this number was multiplied with adipocyte volume to calculate adipocyte weight. To determine adipocyte number, adipocyte mass was divided by the adipocyte weight.

3.3.2 Lipolysis

Isolated adipocytes were suspended in Krebs-Ringer phosphate buffer (pH 7.4) and bovine serum albumin (20mg/ml), ascorbic acid (0.1mg/ml) and glucose (1mg/ml). They were incubated with or without various concentrations of isoprenaline, ANP, dobutamine or terbutaline in a shaking water bath set at 37 °C for two hours. Afterwards samples were placed on ice to stop lipolysis. An adipocyte free aliquot was taken for glycerol measurements. Glycerol concentrations in absence of lipolytic stimulating hormone are considered basal lipolysis and glycerol concentrations with highest concentration of lipolytic agent are termed stimulated lipolysis. Glycerol is measured since adipocytes lack glycerol kinase and thus cannot reuse glycerol. Lipolysis can be expressed per cell, per gram lipid or as a ratio of stimulated over basal lipolysis.

3.3.3 Microdialysis

Microdialysis is a minimally invasive sampling technique that permits both delivery of an agent into tissue and measurement of any free unbound analyte from the extracellular space. In Study IV microdialysis was used to assess *in situ* lipolytic response to ANP. Lidocaine (200 ul 1%) was injected subcutaneously ten cm laterally of the umbilicus in a cohort of 16 men (seven lean and nine overweight), after which a Microdialysis probe (Carnegie Medicine, Stockholm, Sweden) connected to a microinjection pump (Harvard apparatus, Les Ulis, France) was inserted. The probe membrane has a size of 20 x 0.5 mm and a 20-kDa molecular-weight cutoff.

Ringer solution (139 mmol l^{-1} sodium, 2.7 mmol l^{-1} potassium, 0.9 mmol l^{-1} calcium and 140.5 mmol l^{-1} chloride) containing 10 μmol l^{-1} hANP (human α-ANP1-28) (Clinalpha, Laüfelfingen, Switzerland) was infused together ethanol supplementation, to estimate changes in WAT blood flow around the probe, using an ethanol escape method [137]. First only a ringer solution with ethanol was infused but after a 60-minute equilibration period hANP was added for 60 minutes. 15-minute fractions were collected during both infusion and recovery period. Ethanol in both perfusate and dialysate was analyzed with an enzymatic

method [138]. Glycerol in dialysate was determined with an ultrasensitive radiometric method [139].

3.3.4 Western blot

For identifying alterations in the ANP lipolytic signaling cascade in Study IV, subcutaneous adipose tissue protein was isolated and analyzed in a subgroup from cohort 1 (16 non obese and 27 obese subjects). In 200-600 ul RIPA buffer and protease inhibitor cocktail set V (Calbiochem EMD Chemicals, Darmstadt, Germany) a small amount of adipose tissue (100-300mg) was homogenized (IKA T10 basic ULTRA-TURRAX, IKA-Werke Chemicals, Darmstadt, Germany) on ice. Lysates were centrifuged at 14000 rpm for 20 minutes at 4 °C. The infranatant was transferred to new tubes and protein concentrations were determined using Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

On 10% polyacrylamide gels, 50 µg of protein was separated overnight and then blotted onto polyvinylidene difluoride membranes (Amersham Hybond, GE Healthcare, Buckinghamshire, UK) using electroblotting. Membranes were then washed in Tris-buffered saline with 0.1% Tween 20 (TBS-T), 0.35 M NaCl and blocked with TBS-T containing 3% ECL Advance Blocking Agent (GE Healthcare) for one hour. Overnight at 4 °C membranes were incubated with primary antibodies and blocking solution. Next day, following repeated wash steps, blots were incubated with secondary antibodies at room temperature for one hour. Antibodies used were primary; α-NPRC (Sigma-Aldrich, St Louis, MO, USA; SAB2501867, 1:1000 dilution); α-NPRA (Abcam, Cambridge, UK; ab154266, 1:1000 dilution); α-PDE5A (Santa Cruz Biotechnology, Inc., Dallas, TX, USA; SC-32884, 1:500 dilution); α-cGKI (Abcam ab90502, 1:1000 dilution); and α-beta actin (SigmaAldrich A2066, 1:2000 dilution) and secondary were horseradish peroxidase-conjugated-IgG or α-goat or α-rabbit (Sigma-Aldrich). To avoid bands overlapping with background signals membranes were incubated with one primary antibody at a time, in the order listed above. Finally, membranes were washed with TBS-T and chemiluminescence (Amersham ECL Advance Western Blotting Detection Kit, GE Healthcare) was used to detect protein in a Chemidoc XRS system (Bio-Rad Laboratories, Hercules, CA, USA) using Quantity One software.

3.3.5 RNA sequencing

In order to further characterize adipose tissue phenotype we analyzed gene expression by RNA sequencing in Study II and III. We utilized cap analysis gene expression (CAGE), an RNA sequencing technique.

Adipose RNA was extracted with RNeasy Lipid Tissue Mini Kit (QIAGEN). RNA concentrations were measured (Nanodrop NP-1000) and quality determined by a bioanalyzer RNA 6000 Pico Kit (Agilent Technologies). Next CAGE libraries were prepared by reverse transcribing mRNA to cDNA, adding a linker and amplifying transcripts by PCR and then sequenced using Illumina Hi-Seq 2500 or 2000 [140]. Sequenced reads were mapped to the human genome using Bowtie [141]. Output is tags per million, i.e., how many individual

transcripts from every specific gene that is found in the sample. Results were normalized and genes with low expression counts excluded.

In Study III we analyzed insulin induced transcriptomics. Here, gene expression was compared in two different biopsies, one taken during fasting and the next after a two-hour insulin infusion during the hyperinsulinemic euglycemic clamp. Gene expression was then compared between fasting and hyperinsulinemic samples and a gene was considered to be regulated by insulin if there was a significant increase or decrease in expression between conditions.

3.4 ETHICAL CONSIDERATIONS

Research always holds the promise of alleviating or even curing disease. However, most research offers this hope only for future, not current patients. Thus, any risks to subjects taking part in research must be limited.

Subjects enrolled in the clinical trial these results stem from, voluntarily agreed to take part and signed an informed consent. The trial underwent ethical review and was granted permission to proceed by a hospital appointed multidisciplinary board. The trial adhered to the principles outlined in the Declaration of Helsinki. Nevertheless, subjects in the trial were exposed to several risks.

Firstly, subjects underwent a DXA examination which exposes them to radiation. The amount of radiation is however equal to only one day of background radiation which in practice can be considered harmless. A permit was granted from a local authority (Strålsäkerhetsmyndigheten) for subjects to undergo the DXA examination.

Subjects also underwent one or several subcutaneous adipose tissue needle biopsies. This yields a local hematoma and some discomfort after the effect of local anesthesia dissipates. There is also a risk of infection or in theory more extensive bleeding, however our clinical research unit has now taken well over 2000 biopsies without any major complication.

Blood testing was also done, with the potential of uncovering previously unknown disease or ambiguous results in need of monitoring or more extensive testing. We took steps to follow-up any abnormalities by having a medical doctor review all results and referring for future care if needed.

Also, by agreeing to take part in a study, medical information is revealed and stored. To ensure anonymity all subject data was coded and kept separate from any personally identifying information.

Finally, individuals have the right to at any time, and without question, withdraw from the trial and have their information and results disposed of.

3.5 STATISTICS

Unless otherwise stated, all values in this thesis and included studies are presented as mean ± standard deviations. Normal distribution was assessed by Shapiro-Wilks's test.

Study I. All variables were compared with simple linear regression to PWV. Analysis of covariance was performed for the effect of gender on the relationship between visceral or subcutaneous adipocyte volume and PWV. Also, the relationship between either visceral or subcutaneous adipocyte volume, and PWV (set as the dependent variable) and another single variable were determined by multiple regression. Finally, a multiple regression model was performed with PWV as the dependent variable and stepwise removal of non-significant regressors.

Study II. Variables before and after weight loss were, if normally distributed, compared with paired-sample Student t test (two-tailed), and, if non-normally distributed, the related-samples Wilcoxon signed–rank test. Baseline adipose and metabolic variables were set as independent variables and single regression was performed with changes in PWV as the dependent variable. Finally multiple regression analysis with either subcutaneous adipocyte volume or expression of *COL41A* set as the independent variable and PWV as the dependent variable were done. Models were adjusted for known determinants of arterial stiffness.

Study III. Variables were compared using unpaired Student's t-test (two-tailed).

Study IV. Variables between obese and non-obese individuals were compared with unpaired Student's t-test. Paired Student's t-test was used when comparing obese subjects before and after weight loss. When assessing glycerol response in the microdialysis experiment, a two-way repeated measure of analysis of variance was done. Finally, the relationship between hormone induced lipolysis and clinical variables was assessed by spearman rank correlation, and multiple regression. A power calculation was performed indicating 80% power to detect a 25% difference between the groups if at least 31 subjects were included in each group.

4 RESULTS

Detailed results are available in each respective paper and manuscript. The most notable results are summarized here.

4.1.1 Study I

Arterial stiffness has been found to be increased in obese individuals, but it is more prominent in individuals with central as opposed to a peripheral accumulation of adipose tissue [124, 142]. Furthermore, an increase in individual adipocyte size is correlated to insulin sensitivity and risk of developing type 2 diabetes [143-145]. Moreover, specific increase in visceral adipose cell size is linked to dyslipidemia, while the same phenotype in subcutaneous adipose tissue correlates with insulin sensitivity [146]. We explored the association between PWV and adipocyte size and number in both the visceral and subcutaneous adipose depot in a cohort of 120 obese individuals. Subjects underwent extensive phenotyping (see above methods section).

Average cohort aPWV was 8.13 ±1.9 m/s. Individual associations between clinical variables and PWV are displayed in Table 2.

Table 2. Clinical characteristics and correlation with arterial stiffness.

Variable	*Data available in* n *individuals*	*Nr or mean +S.D. (range)*	*r-value*	*P-value*
Gender (male/female)	120	21/99	N/A	0.12
Age, years	120	43 ±10 (18-62)	0.43	<0.0001
Body mass index	120	39.1 ± 3.6 (32.6-57)	-0.05	0.60
Waist circumference, cm	120	122 ± 9.6 (101-152)	0.26	0.0041
Waist-Hip Ratio	120	0.98 ± 0.07 (0.78-1.15)	0.31	0.0006
Waist-Length Ratio	120	0.73 ± 0.05 (0.59-0.90)	0.15	0.11
Systolic blood pressure, mmHg	120	136 ± 16 (96-180)	0.46	<0.0001
Diastolic blood pressure, mmHg	120	80.2 ± 11 (54-125)	0.48	<0.0001
Resting pulse rate, beats/min	120	68 ±10 (45-99)	0.37	<0.0001
PWV, m/s	120	8.13 ± 1.9 (4.6-18.2)	N/A	N/A
Insulin sensitivity (glucose infusion rate, mg/kg*min)	102	4.48 ± 1.75 (0-7.8)	-0.20	0.045
P-glucose, mmol/l	119	5.6 ± 1.3 (4.4-14.2)	0.10	0.27
P-insulin, mU/l	119	15.6 ± 9.4	0.15	0.10
S-Free fatty acids, mmol/l	119	0.70 ± 0.25 (0.2-1.4)	-0.04	0.69
P-total cholesterol, mmol/l	120	4.85 ± 1.0 (2.5-9.4)	0.19	0.039
P-HDL, mmol/l	120	1.2 ± 0.29 (0.6-2.0)	-0.077	0.40
P-triglycerides, mmol/l	120	1.39 ± 0.65 (0.4-3.6)	0.23	0.010
Total body fat, kg	119	53.7 ± 9.5 (36.6-90.1)	0.037	0.69
Subcutaneous fat mass (sWAT), kg	107	3.17 ± 0.9 (0.78-6.2)	-0.13	0.19
Visceral fat mass (vWAT), kg	107	2.2 ± 1.0 (0.82-5.3)	0.38	<0.0001
Subcutaneous fat cell volume, picolitres	120	878 ± 199 (476-1396)	0.20	0.031
Visceral fat cell volume, picolitres	119	569 ± 191 (167-1188)	0.45	<0.0001
Subcutaneous fat cell number (x 109 cells)	107	4.11 ± 1.2 (1.3-7.9)	-0.26	0.006
Visceral fat cell number (x 109 cells)	107	4.34 ± 1.5 (2.4-9.2)	-0.26	0.36

Abbreviations: HDL, high density lipoprotein; N/A, not applicable; P, fasting plasma; PWV, pulse wave velocity; S, serum; s.d, standard deviation; WAT, white adipose tissue. Values are given as actual numbers of subjects or mean ± s.d. as well as range. Student's t-test was used for nominal variables. For continuous variables, linear regression analysis was used; *r*- and *P*-values are indicated.

Table 2. Clinical characteristics and their correlation with PWV in *Study I*

The study confirmed several previous known associations between PWV and vascular parameters such as systolic and diastolic blood pressure and pulse rate. We also confirmed correlations between measures of central adiposity, waist hip ratio and visceral fat mass and PWV, while no such significant associations existed for BMI, total body fat or subcutaneous fat mass. Regarding adipocyte volume, a positive association was found for both visceral and subcutaneous adipocyte volume. To explore this relationship further and adjust for confounders we ran a multiple regression model (Table 3) including all factors that associated with PWV in Table 2.

This demonstrated that, after adjusting for relevant confounders, only visceral adipocyte volume associated significantly with PWV.

Table 3. Factors contributing to variations in PWV

Term	*Estimate*	*Std Beta*	*Std Beta*[a]	*P-value*
Intercept	-1.54			
Age, years	0.040	0.22	0.047	0.011
Diastolic blood pressure, mmHg	0.043	0.26	0.068	0.0017
Resting pulse rate, (beats per min)	0.047	0.25	0.061	0.0015
Visceral adipocyte volume, (pl)	0.0025	0.25	0.063	0.0020

Multiple regression analysis was performed using PWV as the dependent variable and the factors significantly associated with PWV in Table 1 as independent variables (that is, age, waist circumference, waist-hip ratio, systolic and diastolic blood pressure, pulse rate, insulin sensitivity, P-total cholesterol, and triglycerides, vWAT mass, visceral fat cell volume, and subcutaneous adipocyte volume and number). Step-wise removal of regressors that did not contribute significantly to the model resulted in variables listed in the table. The model had an overall F-ratio of 20.5, P= <0.0001 and R^2=0.42. Estimates, standardized beta coefficients (Std beta), Std Beta[a]. *P*-values are indicated for each individual variable.

Table 3. Factors contributing to variations in PWV in *Study I.* Note, Std Beta[a] is an incorrect term, values in this column are the partial R^2 of each variable.

4.1.2 Study II

Short and moderate term weight loss (< one year) leads to improvement in arterial stiffness [118]. It is still unknown if changes last long term and what adipose tissue factor, if any, can predict improvement. For this study we analyzed 82 subjects before and two years after RYGB surgery, excluding subjects with anti-hypertensive pharmacotherapy and one individual with a faulty PWV measurement.

We found that pronounced weight loss due to bariatric surgery led to a small but lasting improvement in PWV 7.80 ±1.50 VS m/s vs 7.23 m/S ±1.41, p = 0.006). As expected, subjects also improved significantly in all measured metabolic and adipose outcomes.

To find WAT predictors of improvements in PWV following significant weight loss we performed linear regressions between change in PWV (dependent variable) and adipose and metabolic variables (independent variables). Results are displayed in Table 4, and after correcting for multiple testing, only subcutaneous adipocyte volume remained significant.

TABLE 4. Simple linear regression of the association between baseline adipose and metabolic characteristics (set as independent variable) and changes in aortic pulse wave velocity (set as dependent variable) following weight loss. r- and p-values are shown, the latter also after Bonferroni correction (multiplied by 15).

	r-value	p-value	*Bonferroni corrected p-value*
BMI	0.299	0.006	NS
Waist–hip ratio	0.044	0.693	NS
Body fat, %	0.011	0.919	NS
EVAT, g	0.244	0.036	NS
ESAT, g	0.179	0.125	NS
Visceral-to-subcutaneous fat mass ratio (EVAT/ESAT)	0.117	0.316	NS
Subcutaneous adipocyte volume, pl	0.390	0.0003	0.0045
Visceral adipocyte volume, pl	0.109	0.341	NS
Fasting insulin levels, mIU	0.302	0.006	NS
Insulin sensitivity, (glucose infusion rate) mg/kg min^{-1}	0.012	0.921	NS
P-fasting glucose, mmol/l	0.113	0.315	NS
S-CRP, mg/l	0.012	0.919	NS
S-Triglycerides, mmol/l	0.047	0.677	NS
S-FFA, mmol/l	0.091	0.414	NS
LDL cholesterol, mmol/l	0.013	0.907	NS

Abbreviations: BMI, body mass index; CRP, C-reactive protein; EVAT, estimated abdominal visceral white adipose tissue; ESAT, estimated abdominal subcutaneous white adipose tissue; FFA, free fatty acids; LDL, low-density lipoprotein; NS, not significant; P, plasma; S, serum.

Table 4. Relationship between individual factors and changes in PWV.

With subcutaneous adipocyte volume being the only adipose factor able to predict improvements in our cohort, we also wanted to establish if the relationship remained significant after adjusting for known arterial stiffness confounders. Thus, we created two multiple regression models, adjusting for systolic blood pressure and resting pulse rate in model 1 and systolic and diastolic blood pressure, resting pulse rate, age, sex, smoking status, and self-reported exercise in model 2. Results are displayed in Table 5.

TABLE 5. Multiple linear regression of the association between baseline subcutaneous adipocyte volume (set as independent variable) and change in aortic pulse wave velocity (set as dependent variable) following weight loss

	Model 1		**Model 2**	
	Std beta	p-value	Std beta	p-value
Subcutaneous adipocyte volume	0.293	0.008	0.279	0.014

Model 1 adjusted for systolic blood pressure and resting heart rate. Model 2 adjusted for systolic blood pressure, resting heart rate, diastolic blood pressure, age, sex, smoking status and self-reported exercise. Standardized beta-coefficients (Std beta) and p-values for subcutaneous volume are given for the two models.

Table 5. Multiple linear regression between subcutaneous adipocyte volume and PWV in two different models in Study II.

We also performed RNA sequencing on a subgroup of individuals and wanted to investigate if individual WAT gene expression could predict improvements in PWV. After correcting for multiple testing, a global analysis found no significant associations. However, when we used a pre-selected list of genes previously associated with arterial stiffness [147], *COL4A1* could in our cohort predict improvements in PWV following significant weight loss. The

relationship remained significant also after adjusting for the same confounders in the previous model 1 and 2.

In summary, we found that significant weight loss leads to sustained improvements in arterial stiffness and that it can be predicted both by baseline subcutaneous adipocyte volume and specific WAT gene expression.

4.1.3 Study III

Weight loss through bariatric surgery can lead to hypertension improvement and even remission in some but not all individuals [14]. There is also a link between insulin resistance and hypertension [148]. We aimed to identify if there were WAT or metabolic differences between those who improved in blood pressure compared to those who did not, after significant weight loss. We included all hypertensive females with multiple blood pressure readings and available transcriptomic data.

We defined hypertension as having multiple blood pressure readings (at different timepoints) at or above 140 mmHg systolic BP or 90 mmHg diastolic BP, or treatment with anti-hypertensive pharmacotherapy. In order to have access to blood pressure readings at more than one timepoint (i.e., at our research laboratory) we collected data from the surgical outpatient clinics subjects attended as part of their standard medical care before and after their bariatric surgery. In total, 18 women were included, ten who improved after weight loss and eight did not.

To determine which factors could potentially predict blood pressure improvement after significant weight loss we compared multiple variables between the two groups. While the improved group was significantly younger (44±5 vs. 51±7, $p = 0.03$), no other parameter differed between the groups. On a transcriptomic level there were no differences in fasting WAT gene expression between the groups.

To investigate the role of insulin resistance and hypertension, we compared insulin resistance in multiple peripheral tissues between the two groups. Again, there was no difference in skeletal muscle insulin resistance (as exemplified by glucose uptake during the hyperinsulinemic euglycemic clamp) or hepatic insulin resistance (measured by HOMA), with both groups starting at a similar level and improving equally after weight loss in both parameters. However, adipose tissue insulin sensitivity, in this study defined as insulin induced gene expression, displayed large differences with 29 genes regulated by insulin at baseline in the non-improved versus 95 in the improved group. At two years the non-improved group only had 31 genes regulated by insulin despite significant weight loss. This contrasted with the improved group where 185 genes where significantly regulated. Altogether this suggests a model where insulin resistance needs to be alleviated in all peripheral tissues for hypertension to remit or improve.

4.1.4 Study IV

In man there are two primary hormones that stimulate lipolysis: catecholamines and ANP. While catecholamines are well studied, with a blunted response seen in obesity (when measured per g lipid or as a ratio of stimulated over basal) [149], the lipolytic effect of ANP has yet to be studied in obese adipose tissue. To this end we characterized the effect both *in situ* and *in vitro* effect of ANP on adipocyte lipolysis.

For the *in vitro* studies, adipocytes obtained from abdominal subcutaneous needle biopsies from 122 subjects (both obese and non-obese) were subjected to lipolysis measurements either spontaneous (no hormonal stimulation) or after the presence of various lipolytic hormones, i.e., stimulated lipolysis. Fifty-two of the women were reinvestigated two years after bariatric surgery (all available subjects at the time of data collection). Results showed that ANP stimulated lipolysis was decreased in the obese when measuring per g lipid (2.9±1.4 vs 1.6±0.9 p =0.0001) and when expressed as a ratio of stimulated over basal lipolysis (12.1±10.1 vs 5.7±3.5 p =0.0001). Noticeably, unlike isoprenaline stimulated lipolysis, there was no increase in stimulated lipolysis when measuring per cell. Further underscoring the blunting effects of obesity was the increase in stimulated lipolysis after weight loss, with subjects improved in ANP stimulated lipolysis both when measured per g lipid (1.5±0.8 vs 4.2± 2.9 p =0.0001) or as a ratio of stimulated over basal (5.3±2.9 vs 13.3± 7.9 p =0.0001).

In situ glycerol assessments in subcutaneous adipose tissue in lean and overweight men after hANP delivery by microdialysis, demonstrated a lower response in the overweight men compared to lean (Figure 5).

We then assessed if we could determine which part of the lipolytic signaling cascade was altered in obesity. ANP can either bind to a cell membrane receptor NPRA (encoded by the *NPR1* gene) leading to a signaling cascade which induces lipolysis, or to a clearance receptor NPRC (encoded by the *NPR3* gene). Using a previously published dataset of 56 non-obese and obese women [150], we found that the expression of several genes were altered in obesity. In the obese subjects *NPR1* levels were reduced (0.81, false discovery rate 5%) while *NPR3* were increased (1.42, false discovery rate 1%). We went on to corroborate this at a protein level in a subgroup (n=43) of the 122 women and found that NPRA was reduced in obesity and NPRC nearly significantly increased (Figure 6).

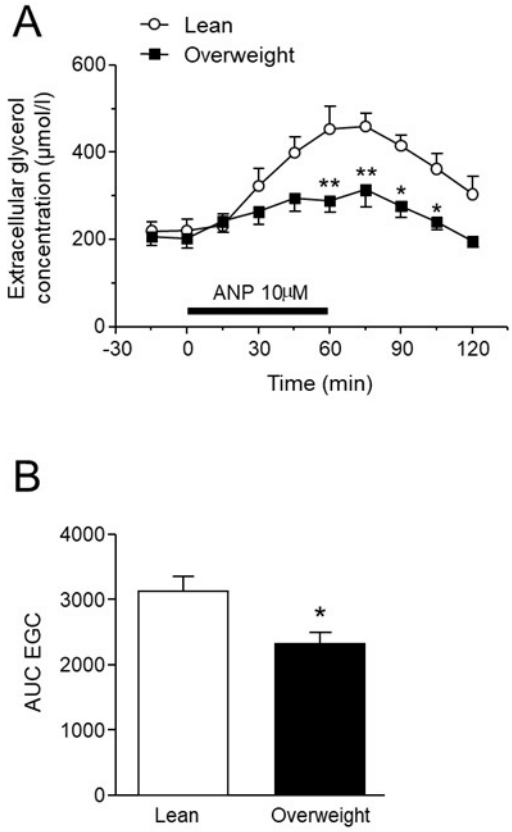


Figure 5. ANP stimulated lipolysis is impaired in subcutaneous adipose tissue in overweight men. A, extracellular glycerol concentrations (EGC) at baseline and after localized infusion of hANP (10µmol/l $^{-1}$, rate 2,5 µl min^{-1}) in abdominal subcutaneous adipose tissue in seven lean and nine overweight individuals. B, Area under curve (AUC) analysis of EGC. Student's T-test was used in both A and B. *p = < 0.05 **p = < 0.01

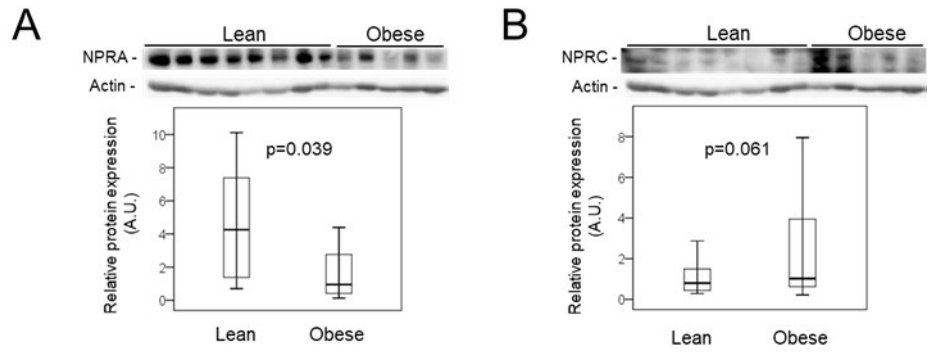


Figure 6. Protein expression of ANP-signaling proteins in human white adipose tissue in lean and obese individuals. A, (upper) representative example of lean and obese protein amount of NPRA and Actin (control). (lower) Quantified NPRA protein concentration of lean and obese subjects. B, (upper) representative example of lean and obese protein amount of NPRC and Actin (control). (lower) Quantified protein concentration of lean and obese subjects. Statistical comparisons were made using Student's t-test (unpaired).

In summary we established that ANP stimulated lipolysis is blunted in obesity and likely this is in part due to altered expression of proteins involved in the ANP lipolytic signaling cascade.

5 DISCUSSION

5.1 STUDY SPECIFIC ASPECTS

5.1.1 Study I

This study confirmed the relationship between central fat mass and arterial stiffness. However, we found that this association was due to visceral adipocyte volume, independently of actual fat mass. It remained significant also after adjusting for other well-known factors that influence arterial stiffness. The study was cross sectional so it's important to note it cannot prove causality but nevertheless mechanisms can be proposed to be investigated in future studies.

Depot specific gene expression may impact on results. The RAAS plays a role in the development of arterial stiffness [151], and visceral adipose tissue secretes most of the components and has pronounced activity of this system [152, 153]. Inflammation is also linked to arterial stiffness [154], and adipocyte size correlates positively with pro-inflammatory TNF-α secretion [155].

FFA have previously been linked to decreased aortic distensibility [156], and large adipocytes are known to have a higher level of spontaneous lipolysis, and thus FFA release [157, 158]. However, it should be noted that during fasting conditions in our study we found no association between serum FFA and PWV.

Finally, insulin resistance associates with arterial stiffness [123] (insulin sensitivity also associated inversely by single linear regression in our study) and both visceral and subcutaneous adipocyte size associate negatively with insulin sensitivity [146, 159]. Furthermore, adipocytes from individuals with arterial stiffness have been shown to have reduced levels of Insulin Receptor Substrate-1 [160], one important enzyme in the insulin signaling cascade.

In summary, several mechanisms may explain the association between visceral adipocyte size and arterial stiffness.

5.1.2 Study II

Study II demonstrated that weight loss led to long term improvements in PWV, with a reduction of on average 0.58 m/s. This change may seem small, but in fact lowered mean PWV to that of a healthy reference population [161]. The effect is also larger than what is seen after exercise interventions [162], and likely is clinically relevant as a rise in PWV by 1m/s increases mortality by 15% [108].

Furthermore, the study again showed that fat mass *per se* does not seem to be an important WAT factor with regards to arterial stiffness, but rather adipocyte size. Here, subcutaneous adipocyte volume was the only metabolic or adipose variable that predicted improvements in PWV following significant weight loss. This is in line with a meta-analysis finding no

correlation between baseline weight and reductions in PWV following weight loss [118]. Many of the phenotypic changes seen in larger adipocytes that could potentially impact on PWV were outlined above. In addition, larger subcutaneous adipocytes may be indicative of an inability to further expand the subcutaneous adipose tissue depot, resulting in ectopic lipid deposition [163] which can occur along the arterial wall, potentially leading to arterial stiffening [112].

Interestingly, subcutaneous WAT *COL4A1* expression was also independently linked to reductions in PWV following weight loss. This collagen alpha chain, which makes up basement membranes of blood vessels, has previously been linked to arterial stiffness in both genome wide association studies [164] and, in lymphoblastoid cell line gene expression [147]. Thus, it is unclear if this constitutes an adipose tissue specific or more general marker of arterial stiffness. There was no association between subcutaneous adipocyte size and *COL4A1* expression indicating that they may affect or be related to arterial stiffness through different mechanisms.

5.1.3 Study III

While insulin resistance is known to influence BP, this was the first study to show increased insulin sensitivity in adipose tissue in obese who improved in blood pressure following weight loss. Skeletal muscle insulin resistance is a well-known feature of hypertension [165] and results suggest that insulin resistance needs to abate in all tissues or hypertension will remain despite weight loss.

We also investigated (currently not in this version of the manuscript) the insulin induced WAT gene expression in a group of eight normotensive never obese controls (matched with the non-improved group for anthropometric measures including BMI and age, skeletal muscle -, and hepatic insulin sensitivity) and found them to have the highest number of regulated genes (n=358), further suggesting a relationship between WAT insulin resistance and blood pressure.

As there were no differences in fasting gene expression, it is unlikely that the WAT phenotype differs regarding inflammation, hypoxia, or fibrosis. Likely the WAT in the improved group is more dynamic but burdened by obesity, and the effect of any anti-hypertensive factors is suppressed but ameliorates with weight loss. Genes selectively regulated in both the improved and never obese groups includes several which have previously been linked to BP: *ADAMST1* (beneficial vascular wall remodeling) [166], *DUSP4* and *HOXA9* (nitric oxide production) [167, 168], *SESN2* (redox balancing/endothelial health) [169], and *NOTCH1* and *HES1* (arterial remodeling and homeostasis) [170, 171].

5.1.4 Study IV

This was the first study that investigated the lipolytic effect of ANP in obese humans. We found that, similar to catecholamine induced lipolysis, ANP stimulated lipolysis was blunted

but this was reversed after weight loss. Furthermore, an *in situ* microdialysis experiment with locally administered hANP also showed a blunted lipolytic response in overweight compared to lean men. Alterations in the signaling cascade with a lower WAT amount of receptor NPRA and a trend towards an increased amount of clearance receptor NPRC may explain the altered response. Our results, including changes in NPRA receptor expression have subsequently been corroborated in another study [172]. Recently it was also shown that catecholamine stimulated lipolysis in obesity is diminished due to a downregulation of its cell-surface receptor, in part due to pro-inflammatory factors like TNFα [173]

Changes in lipolysis have systemic effects and a high basal and low catecholamine stimulated lipolysis associate with weight gain [174], while an increase in both basal and stimulated catecholamine lipolysis are linked to higher cardiovascular risk scores, but interestingly no such association is seen for ANP stimulated lipolysis [157]. Low levels of ANP correlate with increased risk of developing diabetes [175], and while we found associations between ANP lipolysis and various measures of insulin sensitivity these associations became non-significant when adjusting for BMI. In summary, while ANP stimulated lipolysis is dysregulated in obesity, it is still unclear what specific pathological consequences this may incur.

5.2 LIMITATIONS

None of the study designs in this thesis can provide causality, as they are not randomized but observational by design.

The NEFA cohort includes mainly women of Caucasian descent (personal observation, we did not collect data on ethnicity), limiting generalizability of our results with regards to men and other ethnicities. As we recruited the cohort from surgical clinics from individuals who voluntarily agreed to undergo bariatric surgery there were few men available for inclusion, as they less frequently opt for the procedure. We handled the low number of men differently in the studies: they were excluded in *Study III* and *IV* and included in *Study I* and *II.* In *Study I* there were some differences in visceral adipose characteristics between men and women, but we performed an analysis of covariance to control for the influence of gender, which was non-significant. Nevertheless, more studies with larger cohorts of men would be needed to confirm that associations between our variables and outcomes are true also for males. The same applies for being able to generalize our results to other ethnic groups.

Our subjects lost weight by bariatric surgery. This method of weight loss may induce specific metabolic changes that may not be seen by diet and exercise achieved weight loss [176].

In *Study I and II* we use a brachial oscillometric method to measure PWV instead of the gold standard direct (through an intravascular catheter) or indirect (by use of mechanotransducers or tonometers placed directly on the carotid and femoral artery) technique to measure PWV. While cardiovascular outcome studies do not currently exist for the oscillometric method, methodological papers found good reproducibility and validity in comparison with direct [177] and gold standard carotid-femoral [178] measurements. Importantly we used the same

method for our follow-up two years later. We also took care to follow all expert recommendations for measuring PWV [107]. Furthermore, we also used a non-validated scale to capture self-reported exercise (included in *Study II and III)*, it's possible that a different, well established, questionnaire may have yielded other results.

Depot specific differences affect adipose tissue phenotype [23]. In *Study II and III* we use gene expression to characterize the adipose tissue and correlate these findings to outcomes. Here it is important to note that we have only sequenced the subcutaneous adipose tissue depot and different adipose tissue depots may have varying and different gene expression. Depot differences also exist with regards to catecholamine stimulated lipolysis [179] and it would be of interest to perform the same investigations in *Study IV* in visceral adipose tissue to determine if similar differences exist in ANP stimulated lipolysis.

Finally, *Study III* undertook a secondary analysis of the cohort, i.e., none of the pre-defined outcomes from the clinical trial. Had we recruited specifically for a BP study we likely would have had a larger cohort and examinations specifically tailored to the outcome.

6 CONCLUSIONS

Specific WAT factors predict and correlate with important changes in the circulatory system, namely:

- Visceral adipocyte volume is the only WAT factor that is independently associated with PWV (*Study I)*.
- Subcutaneous adipocyte volume and WAT *COL4A1* expression predict improvements in PWV following significant weight loss *(Study II)*.
- Subcutaneous WAT insulin sensitivity is higher in subjects who improve in blood pressure following significant weight loss *(Study III)*.

and

- ANP stimulated lipolysis is blunted in obesity, likely due to a downregulation in ligand binding receptors. *(Study IV)*.

7 POINTS OF PERSPECTIVE

This thesis provides a starting point for future research in several areas.

Study II established that weight loss led to sustained reductions in PWV, however the cohort had just a slightly raised PWV. To further inform clinical decision making it would be interesting to perform two more trials. Firstly, to follow the current cohort with a matched obese group who did not undergo surgery and has a similar baseline PWV as the intervention group. We could then establish if weight loss protects against worsening PWV with increasing age. Secondly, to recruit an obese cohort with a high baseline PWV (>10-12 m/s) and measure the effect of significant weight reduction (by surgery, pharmacotherapy and/or lifestyle intervention).

All sequencing in these studies was done in bulk, which cannot capture the potential heterogeneity of cell types in tissue. It is possible that a different make up or proportion of immune cells may have impacted on results. Mature white adipocytes have also been shown to display heterogeneity, with markedly different responses to insulin [180]. This heterogeneity could influence adipose tissue response to lipolysis, as well as potentially have systemic effects on blood pressure and arterial stiffness. Prospective weight change studies designed and recruited for these outcomes with detailed sequencing would be of interest, but expensive considering current methodology costs.

Less expensive, but of interest, would be a study investigating the effect of adipose tissue insulin sensitivity and blood pressure. Sampling adipose tissue and subjecting *ex vivo* tissue pieces or isolated mature adipocytes to direct traditional insulin sensitivity experiments in hypertensive and normotensive obese cohorts, would in a cross-sectional setting shed further light on the association between adipose tissue insulin sensitivity and hypertension. This would ideally be combined with a hyperinsulinemic euglycemic clamp to assess skeletal muscle insulin sensitivity. Cohorts would then be followed after weight loss and any changes in circulatory outcomes, with a repeat of subject phenotyping.

Furthermore, as mentioned previously, adipose tissue depot specific difference exists. Perivascular adipose tissue, with the ability to exert paracrine effects due to its proximity to vessels, has been shown to have a direct impact on the circulatory system [181, 182]. It would be of special interest to sample and phenotype this depot. Accessibility, sampling must be done during surgery, limits available study design, but cross-sectional studies would still be of interest, and potential results could be followed up and mechanistically investigated in animal models.

Finally, a question which is adjacent to this thesis but nevertheless of interest, is if we should implement arterial stiffness measurements in clinical practice. It is an independent treatable risk factor for cardiovascular mortality that is currently never screened for in Swedish clinical practice. However, any health gains from implementing this method would have to be weighed against the cost of educating healthcare staff in the use and interpretation of different

devices, as well as establishing treatment guidelines and follow-up procedures. Currently at least 25% of individuals with hypertension, an even stronger risk factor for cardiovascular mortality and morbidity, remain undiagnosed in Sweden, indicating that the time may not be right for implementing another vascular measurement. A middle of the road option would be for clinicians to use pulse pressure, which is readily available with just a normal BP measurement. Measuring arterial stiffness by PWV has a greater prediction value compared to PP [183], however PP still has been found to be a predictor of CVD even in normotensive subjects [184] and a stronger predictor of CVD death compared to systolic BP in hypertensive cohorts [185-187]. Identifying populations with increased arterial stiffness may help inform treatment choice both with lifestyle interventions as well as potentially pharmacotherapy choices, as clinical trials have shown differential effect on arterial stiffness despite similar reductions in BP [188, 189], however first line drug choice for hypertension have all been found to reduce arterial stiffness [190].

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